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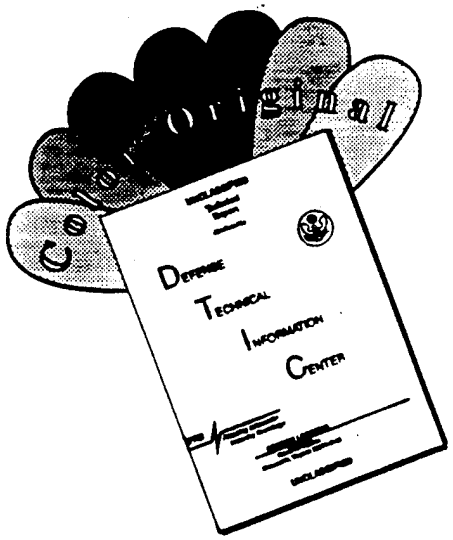

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1. Using T47D cells, our lab built a system in which transfection of FGF4 into this cell line allows cells to form progressively growing tumors in athymic nude mice. The overexpression of FGF also forms lymph node metastasis. Both estrogen and progesterone stimulate the tumor growth. This hormone responsible T47D-derived tumorigenic and metastatic model will be valuable in evaluation of the biological relevance of 80kDa proteinase in breast cancer progression. **2.** We recently cloned an apparently new TIMP (tissue inhibitor of metalloproteinase) named TIMP4 and examined its expression in normal, benign and malignant human breast tissues. Our results demonstrated an inverse correlation between the TIMP4 expression and breast cancer progression. In addition, *in situ* hybridization detected the TIMP4 transcript only in the stromal cells of normal and benign breast tissue but not in the stromals of infiltrating breast carcinomas. Our data suggest that the decreased levels of TIMP4 seen in the breast carcinomas may be too low to prevent MMP activity and cellular invasion. We **hypothesize** that the down-regulation of TIMP4 contributes human breast cancer progression and correlate directly with clinical aggressiveness of breast cancer.

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Adjustment and Introduction

Metastatic dissemination is the primary cause of death for most breast cancer patients. The research effort in PI's lab is to uncover the mechanisms whereby breast cancer undergoes malignant progression and becomes metastatic. Breast cancer metastasis is proposed to depend upon process involving angiogenesis, tumor cell adhesion, motility and proteolysis of extracellular matrix. A general aspect of malignant neoplasia is the capacity to invade and degrade tissue barriers, such as basement membrane, by enhanced proteolysis. We are interested in two major areas: (1) matrix metalloproteinase (MMP); (2) tissue inhibitors of metalloproteinase (TIMP); (3) hormonal regulation of breast cancer progression.

The current Career Development Grant was initially awarded to study the novel 80kDa matrix degrading proteinase in breast cancer progression. While we have made some progress, overall we are not satisfied with the progress of this project. We have devoted many effort on raising monoclonal antibodies to the 80kDa proteinase in attempt to using these antibodies for purification and molecular cloning. Although we obtained the antiserum which can immunoprecipitate the 80kDa proteinase, no success has been met for development of monoclonal antibodies. Based on this unsatisfied work on the 80kDa proteinase, PI has made some adjustment on his effort. Within the same research area of matrix proteinases and breast cancer metastasis, We recently identified and cloned, in collaboration Dr. J. Greene at Human Genome Science, Inc., an apparently new TIMP (tissue inhibitor of metalloproteinase) and examined the expression of this new member in normal, benign and malignant human breast tissues. We designated this novel tissue inhibitor of metalloproteinase as human **TIMP-4**.

Matrix metalloproteinase in breast cancer invasion and metastasis. The proteolytic processes of metastases are thought to initially depend upon type IV collagenase, a metalloproteinase activity encoded by two distinct genes. 72kDa (MMP-2) and 92kDa (MMP-9) enzymes have been described in breast cancer [1]. Correlative evidence for the involvement of type IV collagenase in the invasive phenotype have been demonstrated in a variety of different human cancer systems particularly in human breast cancers [1]. Nonetheless, measurements of the MMP-2 in breast and colon cancer are developing as useful prognostic indicators [1,2]. Immunohistochemistry demonstrated that ductal epithelial cells stain positive for MMP-2 in carcinoma *in situ*, as do the majority of invasive breast carcinomas and metastatic lymph node deposits [3,4]. Immunohistochemical staining has also suggested a prognostic role of MMP-2 for local recurrence of the breast cancer [2]. *In situ* hybridization revealed a strong MMP-2 and MMP-9 mRNA expression in numerous fibroblasts in the stroma surrounding the invasive human breast carcinomas [5]. A major potential application of MMP studies is in the area of detection and monitoring by serum or plasma assay tumor onset, response to therapy and relapse. Indeed, accumulation of procollagen I carboxyterminal propeptide fragments has been reported to be a useful, blood-borne marker of osteoblastic bone metastasis [6]. Detection of MMPs themselves may be even more useful [7,8]. It is of interest that elevated levels of MMP-9, MMP-2, and the MMP inhibitor, TIMP-2, have been detected in the plasma of patients with breast, lung, bladder or colon cancer [9,10].

The another group of enzymes of the matrix metalloproteinase gene family which have been

suggested to be involved in cancer metastases particularly in breast cancer metastasis are the stromelysins which include stromelysin 1 and 2, matrilysin, and most recently, stromelysin 3. Data on the recently described stromelysin 3 suggests that its expression is associated with human breast cancer progression [11,12]. In these studies, stromelysin 3 gene expression was localized to the stromal cells surrounding invasive, but not in situ, human breast carcinoma. The substrate specificity of stromelysin 3 has yet to be defined. Regulation of its activity by other proteinases and particularly by steroid hormones will be an extremely interesting future study.

Much recent attention has been focused on cell membrane associated matrix-degrading proteinases and their role in the activation of pro-MMPs [13-15]. It is thought that matrix metalloproteinases may function in invasion after binding to and being activated by cell surface extensions termed "invadopodia" on invasive tumor cells [13,15]; and such "receptors" are sensitive to metalloproteinase inhibitors [13,14]. It is of interest that MMP-2 activation is restricted to highly invasive estrogen receptor-negative, vimentin-positive human breast cancer cell lines, is independent to MMP-2 production, and is associated with metastatic potential [14]. Recently, a new matrix metalloproteinase with a potential transmembrane domain has been cloned [16,17]. Expression of the enzyme on the cell surface induces specific activation of pro-MMP2 in vitro and enhances cellular invasion of the reconstituted basement membrane. By *in situ* hybridization, we [18] and others [17] have demonstrated the localization of MT-MMP in the stromal cells surrounding the invasive breast carcinomas.

New tissue inhibitor of metalloproteinase TIMP-4. The overproduction and unrestrained activity of matrix metalloproteinase (MMP) has been linked to a variety of malignant conversion of tumor cells [19-21]. The down-regulation of MMP may occur through naturally occurring inhibitor proteins, such as tissue inhibitors of metalloproteinases (TIMPs). TIMPs are secreted multifunctional proteins that play pivotal roles in the regulation of extracellular matrix (ECM) metabolism. Based on structural and functional comparisons, these metalloproteinase inhibitors constitute a protein family that in humans is composed of four members, TIMP-1, TIMP-2 [22], the recently cloned TIMP-3 [23,24], and **a novel TIMP described herein designated as TIMP-4** [25]. Their most widely recognized action is as inhibitors of matrix MMPs. Thus, the net MMP activity in the tumor environment is the result of the balance between activated enzyme levels and TIMPs levels. The inactive, unclipped MMPs are generally secreted as a complex with a TIMP. TIMP-1 is secreted with MMP-9, TIMP-2 is associated with MMP-2 and TIMP-3 with matrix [23]. Enzyme activity seems to depend upon other local determinants in the tumor or an imbalance of MMP/TIMP secreted ratios.

Project 1: 80kDa Proteinase

Establishment of metastatic human breast cancer cells. Animal models of cancer have, in the past, principally been useful for studying aspects of carcinogenesis, tumor growth, and responses to therapeutic agents. However, in recent years researchers have begun to more fully appreciate and attempt to model some of the more deadly aspects of the disease: tumor progression, drug resistance, and metastasis. A recent trend has been to implant human tumor cells into their corresponding sites of origin in the nude mouse and to attempt to model appropriate routes of metastatic dissemination. Two advances have begun to make it possible by this approach to model nearly the complete

metastatic pathway of a human breast cancer in nude mice. These advances relate to better tumor vascularization and visualization.

A metastatic breast cancer cells are required for evaluation of biological relevance of 80kDa proteinase in breast cancer invasion and metastasis. As previously demonstrated, hormone responsive metastatic MCF-7 cells can be obtained by utilizing cDNA-mediated transfection of an angiogenic growth factor, FGF-4 [26,27], we therefore have adopted the same approach to try to establish a metastatic T47D-derived cells. The rationales for choosing hormone-dependent T47D cells are based two facts: 1) the 80kDa proteinase was initially identified in hormone-dependent breast cancer cell lines particular in T47D cell; and 2) our initial results demonstrate that the expression of 80kDa proteinase correlate with T47D/T47Dco progression model [28,29]. For this, T47D cells were transfected with the same FGF-4 vector as MCF-7 cells [26]. This eukaryotic expression vector, termed pCNCKSEB, contains the cDNA for FGF-4 under control of the cytomegalovirus (CMV) immediate early gene promotor and a second transcriptional unit conferring G418 resistance under the control of the same promoter. Two clonal cell lines (TKS-7, TKS-2) were characterized. To detect biologically active FGF-4 secretion by TKS cells, a cross-feeding experiment using SW-13 adrenal carcinoma cells were performed as we previously described. As demonstrated in **Table 1**, clone TKS-7 expressed higher levels of FGF-4 than that of TKS-2 cells. The secreted levels of FGF-4 from TKS-7 cells is compatible to previously established metastatic MKL-4 cells.

Table 1

Detection of secreted FGF-4 by transfected T47D clones

Conditioned medium derived from cell line	Colonies of FGF-4-sensitive SW-13 cells (x 10^3)
Cell-free control	4±1
T47D	10±3
TKS-2	32±11
TKS-7	90±21
MKL-4	85±13

T47D cells were transfected by FGF-4 as previously described for MCF-7 cells [26]. A bioassay for FGF-4 secretion using cloning of SE-13 cells as previously described [26,27] was carried out. A high number of SW-13 colonies indicates high secretion of an FGF-related molecule. Numbers are mean plus SD of triplicate determination.

Initial studies have demonstrated that estrogen can induce increased invasive behavior for hormone dependent MCF-7 cells in an in vitro Boyden chamber assay [12,13]. Although extensive studies must be carried out to investigate whether this steroid-induced an increase in invasiveness is due to an induction of type IV collagenase activity, no qualitative or quantitative changes have been detected to date. We have now identified a novel 80kDa matrix-degrading proteinase from hormone dependent breast cancer cells. The likelihood that the 80kDa proteinase may contribute to this hormone regulation of invasiveness need to be answered. We are interested in developing this

hormone responsive TKS-7 cells as model for study the biological relevance of the 80kDa proteinase in breast cancer progression. In this regard, we have begun to test the *in vivo* behavior of TKS-7 cells. The results in **Fig. 1** show that the tumor, following 6 weeks treatments with tamoxifen and progesterone, was very aggressive, sometimes quite bloody (**Fig. 1A, left mouse**). As PI described in another Army grant, the current controversy regarding the progestational effects on breast cancer calls for creation of human model systems whereby progestin- and antiprogestin-sensitive human cell lines are grown as tumors in the nude mouse. A major hurdle has been no progestin sensitive breast cancer cell lines that have been shown to be sufficiently metastatic in the nude mouse to allow testing of progestational and antiprogestational drugs that might interrupt this process. Using our TKS-7 model, We have tested the **hypothesis** that progestational hormones stimulate breast cancer growth and progression; and antiprogestins can interrupt these process. As demonstrated in **Fig. 1**, and **Figs. 2,3 &4**, TKS-7 behaviors like a hormone-independent but still hormone responsive cell line. While estrogen is still a major driving force for TKS-7 tumor growth (Fig. 2), progesterone stimulates the tumorigenesis 5 fold over control (**Fig. 3**). The tumor incidence was also increased from 50% of control group to 70% of progesterone-treated group. We next investigated the effect of antiprogestin onapristone on progesterone stimulated TKS-7 tumor growth. Onapristone alone did not change the basal levels of tumorigenic activities of TKS-7 cells as measured by tumor size and tumor incidence. However, when the antiprogestin pellet was inoculated to progesterone-treated mice, onapristone significantly inhibits the progesterone-induced tumor growth (**Fig. 3**); the tumor size was reduced from 210 mm^3 in progesterone-treated group to 77 mm^3 in progesterone- and onapristone-treated group (64% inhibition); no significant change of tumor incidence was observed. Lymph node metastasis were also examined by histological analysis. No lymph node metastasis was observed in control mice. However, we identified four lymph node metastases in progesterone-treated mice. Furthermore, onapristone treatment resulted in a reduction of progesterone-induced lymph node metastasis (**Fig. 3**). These results are consistent with the previously established metastatic MCF-7 cells showing that the rate of metastasis depends upon time since inoculation and tumor size [26,27].

We are also interested in the effects of antiprogestin alone compared to its effects in combination with antiestrogen in an attempt to test the new endocrine therapy in the tamoxifen relapse setting. The rationale behind this antiestrogen-antiprogestin interaction derives from our assumption that antiestrogen tamoxifen, also a partial estrogen agonist, may increase PR expression in breast cancer cells and therefore enhance their responses to progesterone and onapristone. To test this hypothesis, we first settled out an experiment to investigate the interactions between tamoxifen and progesterone in our TKS-7 model. As demonstrated in **Fig. 4**, a **synergistic** effects of tamoxifen and progesterone was observed in TKS-7 tumor growth. Progesterone alone stimulated tumor growth 4-fold over control; tamoxifen also stimulated tumor growth (2.5-fold), although to a less extent as compared to progesterone, presumably due to its partial estrogenic activity. When the mice were treated with a combination of tamoxifen and progesterone, the resulting TKS-7 tumors were significantly larger than that of tamoxifen alone or progesterone alone. The tumor size was increased from 83.6 mm^3 in tamoxifen-treated group and 134 mm^3 in progesterone-treated group to 259 mm^3 in the combination group. Two representing mice from control non-treated and tamoxifen/progesterone-treated group, showing a dramatic stimulation of tumor growth, were shown in **Fig. 1A**. Picture in **Fig. 5** shows the tumors from control, progesterone-treated, tamoxifen-treated

and progesterone/tamoxifen-treated mice. Such synergistic interaction between tamoxifen and progesterone on TKS-7 tumor growth may due to the tamoxifen-mediated estrogenic effect on up-regulation of PR. We anticipate that the PR levels in tamoxifen-treated mice are increased as compared to that of non-treated or progesterone-treated mice.

To our knowledge, this is the first report on the in vivo stimulation of human breast tumor growth and lymph node metastasis by progestational hormones and the inhibition by antiprogestin onapristone. This information is extremely important in terms of evaluation of consequences of progestin use in contraception, postmenopausal replacement and breast cancer hormone therapy. Currently we are preparing a manuscript for Science. We plan to examine the 80kDa expression in TKS-7 tumors from different hormone-treated mice as we described before [28].

Project 2: The loss of expression of TIMP-4, a new human tissue inhibitor of metalloproteinase, in human breast malignant progression

The net MMP activity in the tumor environment is the result of the balance between activated enzyme levels and TIMP levels. Augmented MMP activity has been associated with the metastatic phenotype of breast carcinomas [20]; the decreased production of TIMPs could also result in greater effective enzyme activity and the invasive potentials [30,31]. One could expect an inverse correlation between TIMPs levels and the invasive potential of malignant tumors. However no such inverse correlation has been demonstrated in breast carcinomas. In contrast, *in situ* hybridization demonstrated a expression of TIMP-1 and TIMP-2 [32,33] in the stromal areas immediately surrounding the invasive breast carcinomas but not in the benign breast tissue. Our recent results also demonstrated the expression of TIMP-3 in both malignant breast epithelial and stromal cells but not in benign breast tissues [34].Within this context, based on the availability of a novel TIMP sequence, we undertook a search for the possible presence of the new human TIMP and its potential biological relevance in breast cancer progression.

Molecular cloning of TIMP-4 complementary DNA. We have searched a database of human genes identified by the expressed sequence tag (EST) method involving automated DNA sequence analysis of randomly selected cDNA clones [35].The sequence of the TIMP-related gene products were searched against approximately 400,000 ESTs using the BLAST program [36], and we revealed an EST from an early human brain library with a strong homology to TIMP-2. An EST from an early human brain library which demonstrated homology to TIMPs was completely sequenced and found to be a partial clone missing sequence at the 5' end. The coding region and 3' untranslated region of this clone was excised from the Bluescript vector by digestion with restriction endonucleases EcoRI and XhoI, and used to make a radioactive probe using the Stratagene Prime-It II kit. The resulting probe was used to screen a Northern blot of total RNA from several human tissues. The highest level of expression was noted in RNA from human adult heart. (**Fig. 6**). Based on this Northern blot data, we screened one million clones from human adult heart cDNA phage library (in the Stratagene Uni-Zap vector) by plaque lift hybridization with the probe as described in Fig. 1. Positive clones were screened for additional 5' sequence by PCR using a vector primer and an antisense primer specific to the partial TIMP-4 gene to assess insert length. The Bluescript vector and insert were excised from the UniZap phage using the Stratagene ExAssist helper phage. DNA

was prepared by alkaline lysis, purified by passage over a Qiagen midicolumn, and completely sequenced using ABI 373a Automated Fluorescent Sequencer protocols. and used to obtain the full-length cDNA sequence

The open reading frame is predicted to encode a 224 amino acids polypeptide. Comparison of the predicted amino acid sequence with the sequences of human TIMP-related proteins is shown in **Fig. 7**. After optimal alignment the putative protein shows 62% sequence similarity with TIMP-1, 50.9% with TIMP-2 and 51.4% with TIMP-3. These calculations do not take into account the significance of any gaps in the alignments. The predicted protein structure of the putative new protein shares several essential features that are characteristic to the TIMP family including completely conserved 12 cysteine residues in the corresponding positions that form intrachain disulfide bonds that fold the protein into a two-domain structure. The presence of a 29-amino acid leader sequence [37], which presumably is cleaved to produce the mature protein, was also observed in the putative new protein. The extensive similarity of the predicted amino acid sequence with TIMPs suggests that the putative new protein is a novel member of the human TIMP family and should be designated as human TIMP-4.

Down-regulation in breast carcinomas. The expression of TIMP-4 in human breast cancers was first investigated in a variety of human breast cancer cell lines (**Fig. 8**). Northern blot analysis failed to detect the TIMP-4 transcript in most breast cancer cell lines except MDA-MD-231 cells which showed a TIMP-4 hybridization pattern similar to the heart with a strong signal of 1.4 kb; a very weak hybridization signal was also detected in MDA-MD-436 cells **(Fig. 8A)**. In order to rule out the possibility of cross-hybridization with either TIMP-1, TIMP-2 or TIMP-3, an additional filter was hybridized with a 390 bp riboprobe which represents a specific nucleotide sequence of the 3'-untranslated TIMP-4. As shown in **Fig. 8C**, the riboprobe recognized the same bands in the RNA from MDA-MD-231 cells as the complete DNA probe, thus confirming that the 1.4 kb transcripts correspond to TIMP-4. The inability to pick up the TIMP-4 mRNA in most breast cancer cell lines by Northern blot suggests a) that the TIMP-4 gene may be only expressed very weakly in breast epithelial cells but is mainly expressed in stromal cells, or b) the expression of the TIMP-4 gene may be down-regulated in breast cancers during the breast malignant progression.

In an attempt to evaluate the biological relevance of TIMP-4 to human breast cancer progression, we conducted a experiment to determine TIMP-4 expression on human breast tumor biopsies. The expression of TIMP-4 on four metastatic breast carcinomas and six benign breast tissues were analyzed by Northern blot. The results in **Fig. 9** demonstrated a downward progression in the levels of TIMP-4 from benign breast hyperplasia or fibroadenomas to the highly metastatic breast carcinomas. Four of the six RNA samples from benign breast tissues showed a strong 1.4 kb TIMP-4 transcript. RNAs from sample B1, a breast hyperplasia, showed two strong hybridization bands at 1 kb and 0.7 kb which suggest that normal or benign breast may be heterogeneous with respect to the TIMP-4 expression or that there is degradation of TIMP-4 mRNA in this sample. A very weak 1.4 kb TIMP-4 transcript was detected in RNA from sample B5. In contrast, either no signal or only a very weak signal of the TIMP-4 transcript can be detected in metastatic breast carcinomas. The existence of TIMP-4 transcripts in human breast tissues and their reduced expression in breast carcinomas indicate a possible role of down-regulation of TIMP-4 in breast cancer progression.

Expression specific to stromal cells of normal and benign breast. In order to localize the cellular source of the TIMP-4 expression and to further assess of the biological relevance of the down-regulation of TIMP-4 expression in breast cancers, we next performed *in situ* hybridization on fixed sections from a variety of different breast samples including 15 carcinomas and 15 benign breast tissues (hyperplasia and fibroadenomas). In these experiments, we examined two aspects of TIMP-4 expression: a) the tissue localization (stromal versus epithelial) of the TIMP-4 and b) the correlation of TIMP-4 expression and breast malignant phenotype. In all cases we found a strong TIMP-4 transcript in the stromal cells surrounding normal or benign breast (**Fig. 10**). The labeling of TIMP-4 mRNA was detectable in the stromal cells in 13 of 15 benign breast hyperplasia and fibroadenomas with the strongest signals in the stromal cells of breast fibroadenomas (**Fig. 10A**). The expression of TIMP-4 was also detected in the stromal cells of normal lobule or duct (**Fig. 10C,D**).

It is striking that in all cases the highly infiltrating malignant breast samples are not labeled either in the neoplastic cells themselves or their surrounding stromal cells (**Fig. 10E**). This is different from our Northern blot which detected low levels of TIMP-4 expression in some breast carcinomas. This apparent discrepancy may have been caused by the small number of samples that were analyzed. Alternatively, it is also possible that TIMP-4 transcripts are expressed at very low levels in a large number of cells of breast carcinomas. In agreement with this possibility, TIMP-4 transcripts could be detected in breast cancer cells by Northern blot (**Fig. 8**).

The high expression of TIMP-4 in the stromal area of normal and benign breast and the loss of TIMP-4 expression in the stromal cells of highly infiltrating breast carcinoma stimulated us to examine the TIMP-4 expression in the low grade *in situ* carcinomas. It is extremely interesting to note that although TIMP-4 signals can be detected in the stromal cells of *in situ* ductal carcinomas, it can only be detected in the stromal cells not immediately surrounding the neoplastic cells; stromal immediately surrounding the neoplastic cells were negative or very weak for the TIMP-4 expression (**Fig. 10F,G,H**). The lose of TIMP-4 expression in the stromal area immediately surrounding the ductal carcinoma may signal or initiate the local invasion of ductal breast cancer cells. This TIMP-4 expression pattern in ductal breast carcinoma is just opposite to the expression pattern of stromelysin-3 which is highly expressed in the stromal immediately surrounding the infiltrating breast cancer cells but only weakly expressed in the central part (the farthest from the neoplastic cells) of the stromal trabeculae [12].

The detection of TIMP-4 expression in benign breast tissues is in remarkable contrast with the previous reports on TIMP-1 and TIMP-2 expressions which have been demonstrated to be mainly located in the peri-tumoral stroma but not in the residual benign breast tissues [22]. We have also analyzed the TIMP-2 and TIMP-3 expression in different human breast tissues in comparison with that of TIMP-4. As shown in **Fig. 11**, in contrast to TIMP-4 expression, higher levels of TIMP-2 and TIMP-3 expression were observed in the areas of malignant breast carcinomas (Fig. 11A & C). Normal or benign breast were stained negatively (Fig. 6B & D). While the TIMP-2 transcript was detected in stromal cells surrounding the invasive breast carcinomas (**Fig. 11C**), TIMP-3 transcript was mainly localized in the malignant breast epithelial cells [**Fig. 11A, ref. 34**]. Breast epithelial cells in normal duct or hyperplasia were negative for TIMP-3 (**Fig. 11B**). The lack of TIMP-4 expression in the breast carcinomas suggests that its down-regulation may mediate the breast malignant progression from benign to metastatic phenotype.

Conclusions

We have described here a novel member of the TIMP family and examined the expression of this new member, TIMP-4, in normal, benign and malignant human breast tissues. Degradation of the extracellular matrix by MMP and other proteinases is a prerequisite for the acquisition of an invasive phenotype by tumor cells. Since TIMPs block the activities of MMPs, the net inhibitory activity of TIMPs might be important in preventing malignant progression from the benign to the metastatic phenotype. In fact, tumor cell invasion and metastasis can be blocked by up-regulation of TIMP expression or an exogenous supply of TIMPs [38]. Similarly, we demonstrated an inverse correlation between TIMP-4 mRNA levels and the malignant nature of breast cancer, which is consistent with the recent demonstration that down-regulation of both TIMP-1 and TIMP-2 contribute significantly to the invasive potential of human glioblastoma [39]. A similar inverse correlation was also demonstrated between TIMP-1 levels and the invasive potential of murine tumor cells [40]. Our demonstration of down-regulation of TIMP-4 in breast carcinomas and its cellular location in the stromal cells of normal and benign breast tissues suggest the role of this new TIMP as one of the permissive factors, when it is down-regulated, in mediating the breast cancer progression. Further studies on the TIMP-4 function will provide potentially promising diagnostic and therapeutic tools in cancer progression.

Reference:

1. Goldberg, G.I. and Eisen, A.Z. *Extracellular matrix metalloproteinase in tumor invasion and metastasis*, In Lippman, M.E. and Dickson, R.B. (eds.): Regulatory Mechanisms in Breast Cancer. Kluwer Academic Publishers, Boston, 421-440, 1990.
2. Tryggvason, K (1993) Type IV collagenase in invasive tumors. Breast Cancer Res. Treat., 24: 209-218.
3. Monteahudo, C., Merino, M.J., San-Juan, J., Liotta, L.A. and Stetler-Stevenson, W.G. (1990) Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am. J. Pathol., 136: 585-592.
4. D'Errico, A., Garbisa, S., Liotta, L.A., Castronova, V., Stetler-Stevenson, W.G. and Grigioni, W.F. (1991) Mod. Pathol., 4: 239-246.
5. Soini, Y., Turskainen, T., Hoyhtya, M., Oikarinen, A., Autio-Harmainen, H. (1994) 72kD and 92Kd type IV collagenase, type IV collagen, and laminin mRNAs in breast cancer: astudy by in situ hybridization. J. Histochemistry and Cytochemistry, 42: 945-951.
6. Francini, G., Bruni, S., Marsili, S., Aquino, A. and Camporeale, A (1993) Procollagen type I carboxy-terminal propeptide as a marker of osteoblastic bone metastasis. Cancer, Epideminology, Biomarkers and Prevention 2: 125-129.
7. Zucker, S., Lysik, R.M., Stetler-Stevenson, W.G., Liotta, L.A., Birkedal-Hansen, H. and Mann, W. (1992) Immunoassay of type IV collagenase/gelatinase (MMP-2) in human plasma. J. Immunol. Meth., 148: 189-198.
8. Moutsiakis, D., Mancuso, P., Stetler-Stevenson, W, Zucker, S. (1992) Characterization of metalloproteinase and tissue inhibitors of metalloproteinase in human plasma. Connec. Tissue Res., 28: 213-230.
9. Zucker, S. (1993) Mr 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. Cancer Res., 53: 140-146.
10. Marguilies, I.M.K., Hoyhtya, M., Evans, C., Stracke, M.L., Liotta, L.A., Stetler_stevenson, W.G. (1992) Urinary type IV collagenase: Elevated levels are associated with bladder transitional cell carcinoma. Cancer Epidemiology, Biomarkers & Prevention, 1: 467-474.
11. Basset, P., Wolf, C., Rouyer, N., Bellocq, J.P., Rio, M.C. and Chmbon, P. (1994) Stromelysin-3 in stromal tissue as a control factor in breast cancer behavior. Cancer, 74: 1045-9.
12. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C. and Chambon, P. (1990) A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature, 348: 699-704.
13.. Strongin, A.Y., Marmer, B.L., Grant, G.A., Goldberg, G.I. (1993) Plasma membrane-dependent activation of then 72kDa type IV collagenase is prevented by complex formation with TIMP-2. Biol. Chem., 268: 14033-14039.
14. Azzam, H.S., Arand, G., Lippman, M.E. and Thompson, E.W. (1993) Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. J. Nat. Cancer Inst., 85: 1758-1764.
15. Monsky, W.L., Kelly, T., Lin, C.Y., Yeh, Y., Steter-Stevenson, W.G., Mueller, S.C. and Chen, W.T. (1993) Binding and localization of Mr 72,000matrix metalloproteinase at cell surface invadopodia. Cancer Res., 53: 3159-3164.
16. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., Seiki, M. (1994b) A matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature, 370: 61-65.
17. Okada, A. et al. (1995) Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. Proc. Natl. Acad. Sci.

92: 2730-2734.

18. **Shi, Y.E.**, Wang, M.S., Xiao, G.W. and Liu, Y.E. Expression of MT-MMP in human breast cancers, in preparation for Cancer Res.
19. E. J. Bernhard, et al., Proc. Nat. Acad. Sci. (1994);C. Pyke, et al., *Am. J. Pathol.* **142,** 359 (1993); M. S. Naylor, et al. *Int. J. Cancer* **58**, 50 (1994).
20. P. Basset, et al., *Nature* **348**, 699 (1990); P. Basset, et al., *Cancer* **74**, 1045 (1994); M. Polette, et al., *Invasion Metastasis* **13,** 31 (1994); M. Polette, et al., *Virchows Arch.* **424**, 6 4 1 (1994). J. M. P. Freije, et al., *J. Biolo. Chem.* **269,** 16766 (1994)
21. A. E. Kossakowska, S. J. Urbanski and D. R. Edwards, *Blood* **77**, 2475 (1991); M. Polette, et al., *Pathol. Res. Pract.* **189**, 1052 (1993); M. S. Naylor, et al., *Int. J. Cancer* **58**, 50 (1994).
22. D. F. Carmichael, et al., *Proc. Natl. Acad. Sci.* **83**, 2407 (1986); L. J. Mullins, et al., *Genomics* **3,** 187 (1988); W. G. Stetler-Stevenson, et al., *J. Biol. Chem.* **265**, 13933 (1990).
23. M. Wick, et al., *J. Biol. Chem.* **269**, 18953 (1994); S. S. Apte, et al., *Genomics* **19**, 86 (1994).
24. J. A. Uria, et al., *Cancer Res.* **54**, 2091 (1994).
25. Greene, J., Wang, M.S., Xiao, G.W., Liu, Y.E.Li, J.Y. and **Shi, Y.E.** The loss of expression of TIMP-4, a new human tissue inhibitor of metalloproteinase, in human breast malignant progression submitted to **Nature**.
26. Kuebayashi, J., Mcleskey, S.W., Johnson, M.D., Lippman, M.E., Dickson, R.B. and Fern, F.G. (1993) Quantitiative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells co-transfected with fibroblast growth factor-4 and lacZ. Cancer Res. 53: 2178-2187.
27. Mcleskey, S.W., Kurebayashi, J., Honing, S.F., Zweibel, J.A., Lippman, M.E., Dickson, R.B. and Kern, F.G. (1993) Development of an estrogen-independent, antiestrogen-resistant and metastatic breast carcinoma. Cancer Res. 53: 2168-2177.
28. **Shi, Y.E.**,Torri, J. Yieh, L., Wellstein, A., Lippman, M.E. and Dickson, R.B. Isolation and characterization of a novel matrix degrading protease from hormone dependent human breast cancer cells. Cancer Res., 53: 1409-1415 (1993).
29. R.B. Dickson, **Y.E. Shi** and M.D. Johnson: A novel matrix-degrading protease in hormone dependent breast cancer. ***Biochem. Soc. Transact.***, 22 (1): 49-52, 1994.
30. R. Khokha, et al., *Science* **243**, 947 (1989).
31. D. T. Denhardt, et al., *Matrix Suppl.* **1**, 281 (1992).
32. D. W. Visscher, et al., *Int. J. Cancer* **59**, 339 (1994);
33. M. Hoyhtya, et al., *Int. J. Cancer* **56,** 500 (1994); R. Poulsom, et al., *J. Clin. Pathol.* **46,** 4 2 9 (1993).
34. **Shi, Y.E.**, Wang, M.S., Xiao, G.W., Greene, J., Liu, Y.E. Expression of TIMP-3 in maglinant human breast carcinomas, submitted to Cancer Res.
35. M. D. Adams et al., *Science* **252**, 1651 (1991); *Nature* **355**, 632 (1992); *Nature Genet.* **4**, 256 (1993); *ibid.*, p. 373.
36. S. F. Altschul et al., *J. Mol. Biol.* **215**, 403 (1990).
37. K. J. Leco, et al., Tissue inhibitor of metalloproteinase-3 (TIMP-3) is an extracellular matrix-associated protein with a destinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem* **269**, 9352 (1994).
38. R. Khokha, *JNCI.* **86,** 299 (1994); R. Khokha, et al., *JNCI* **84**, 1017 (1992); J. E. Testa, *Cancer Res.* **53**, 5597 (1992); O. A. Alvarez, et al., *JNCI* **82**, 589 (1990); T. Y. Sato, et al., *Cancer Res.* **53**, 1397 (1993); Y. A. De Clerck, et al., *Cancer Res.* **52**, 701 (1992).
39. S. Mohanam, et al., *Clin. Exp. Metastasis* **13**, 57 (1995).
40. R. Khokha, et al., *Science* **243**, 947 (1989).

Fig. 1. Growth of TKS-7 cells in nude mice. 5 millions cells were injected into the mammary fat pad of the ovariectomized mice. Animals were scarified 6 weeks after inoculation, and the tumors and lymph nodes were processed for analysis. Nude mice bearing TKS-7 tumors. Right mouse was treated with slowly time-releasing pellets of tamoxifen (5 mg/60 days) and progesterone (10 mg/21 days); left mouse was non-treated control.

Fig. 5. Regulation of TKS-7 tumor growth by steroid hormones. 5 millions cells were injected into the mammary fat pad of the ovariectomized nude mice. The mice were divided into four groups: control (first line); progesterone-treated, 10 mg/pellet/21 days (second line); tamoxifen-treated, 5 mg/pellet/60 days (third line); and progesterone- and tamoxifen-treated (fourth line). Tumors were harvested after 6 weeks. 5 representing tumors from treated groups and 4 representing tumors from control group were placed on the dryice. Although both progesterone and tomaxifen stimulated tumor growth, combined treatment gave the biggest stimulation. Please **notice** that the differences of the real tumor sizes among the different groups are more dramatic than it looks like in the picture because the tumor sizes in the picture only reflect the 2-D measurement. Fig. 10 and Table 2 (next page) also demonstrate such differences.

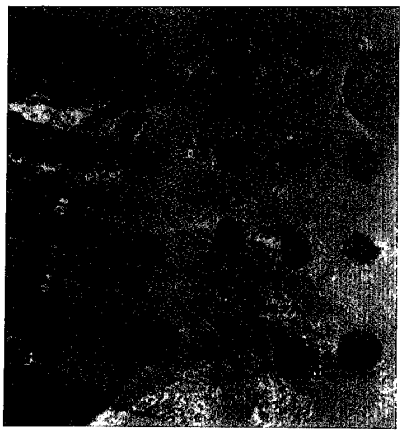
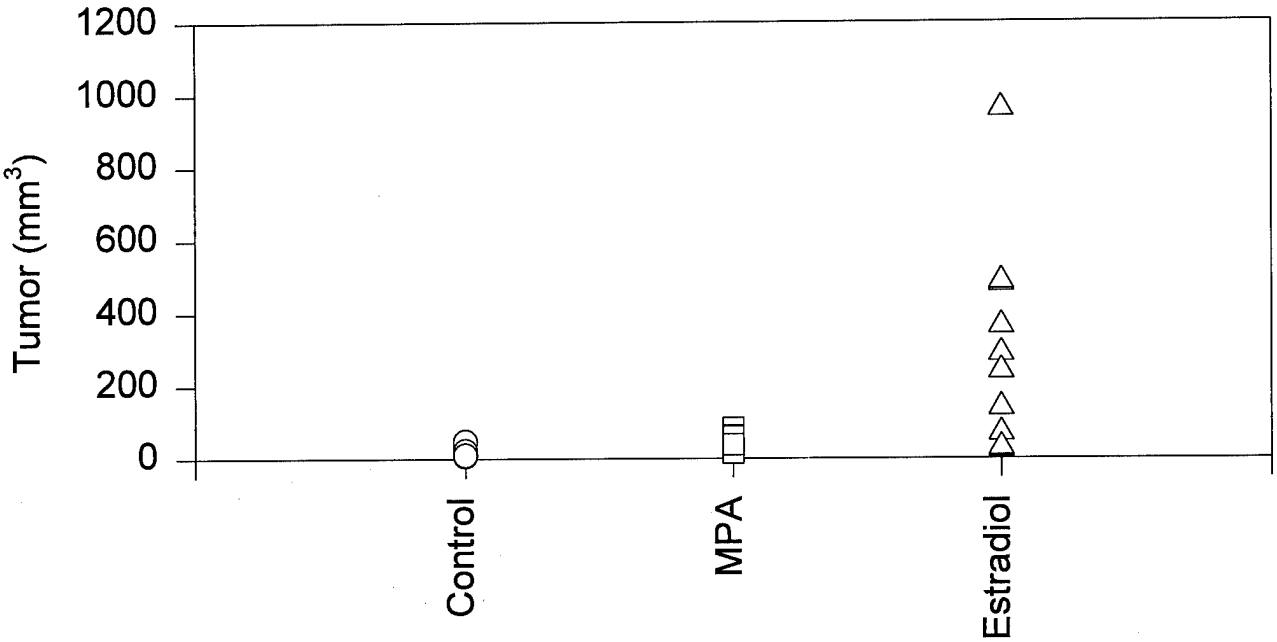


Fig. 2. Hormonal Regulation of TKS-7 Tumor Growth



Effects of MPA and estradiol on TKS-7 tumor growth

	Tumor size (mm^3 + SE)	Tumor incidence (%)
Control	18.8+7.7	50
MPA	47.1+15.7	90
Estradiol	308.2+90.5	100

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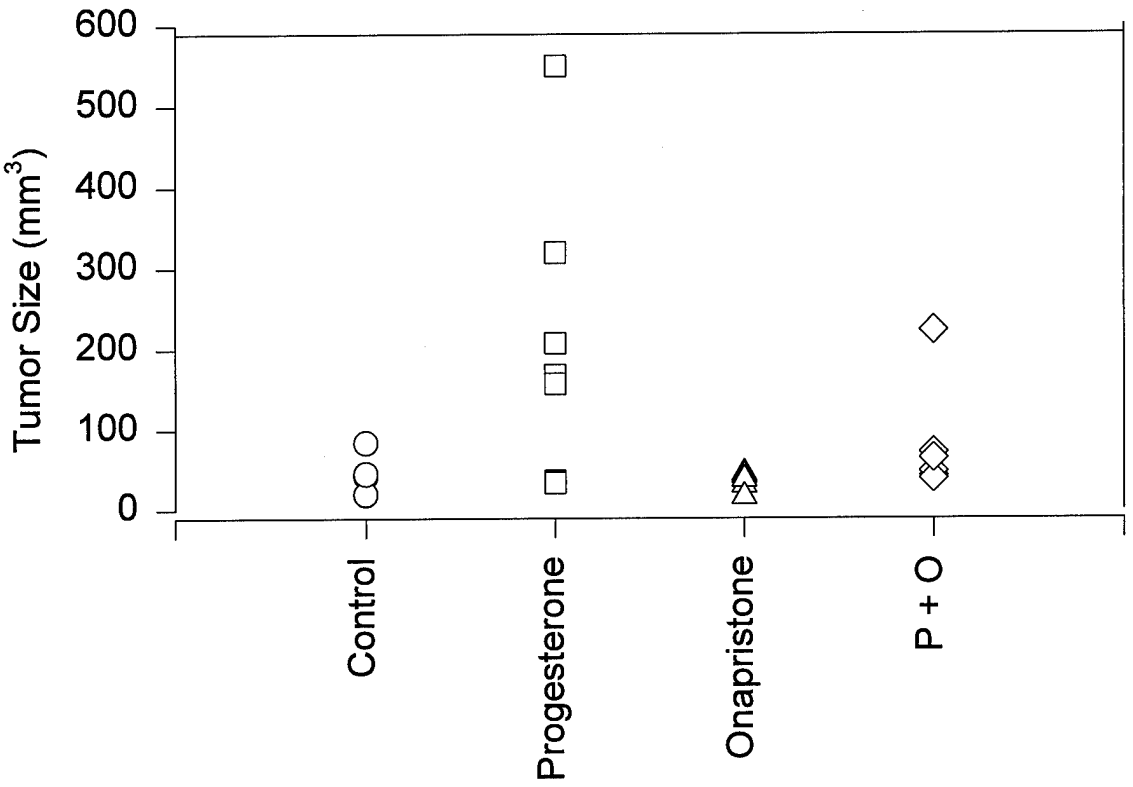
5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. MPA, 1.5 mg/pellet/21 days; estradiol, 0.72 mg/pellet/60 days. The treatments with MPA were replaced every three weeks. Tumors were harvested after 6 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections.

Fig. 3. Effects of progesterone and onapristone on TKS-7 tumor

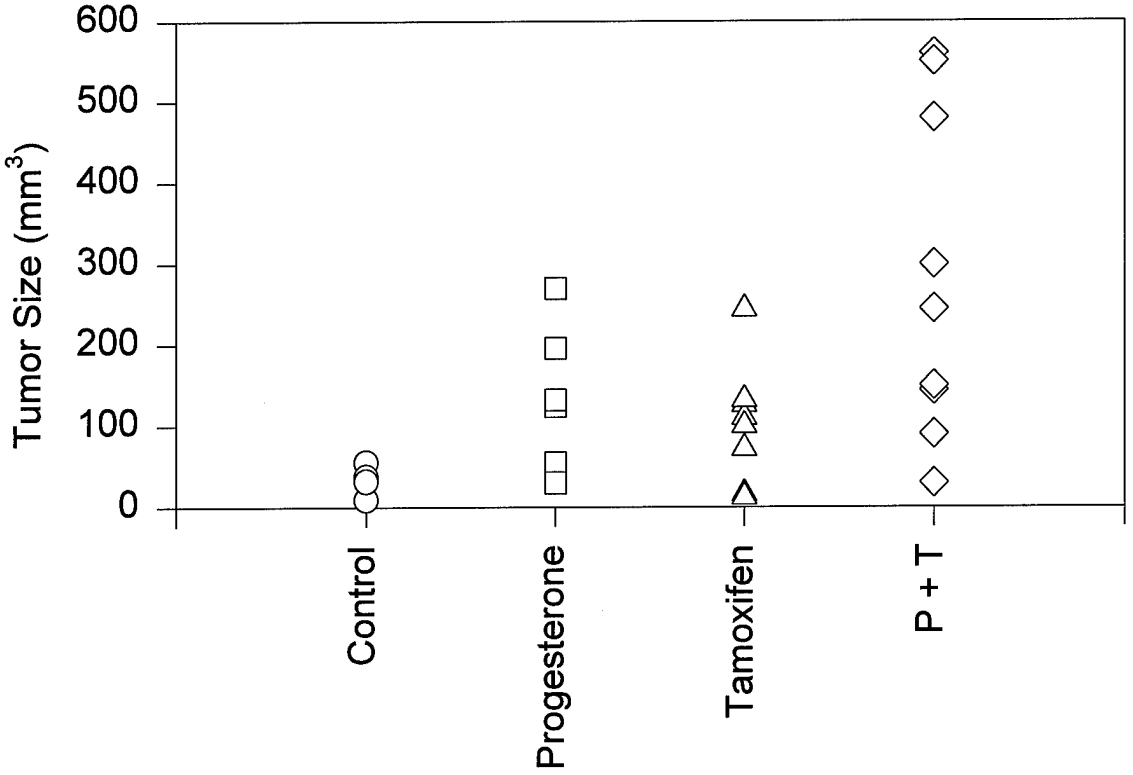


Effects of progesterone and onapristone on TKS-7 tumor growth

	Tumor size (mm^3 + SD)	Tumor incidence (%)	Lymph node metastasis
Control	41.9+11.8	50	0
Progesterone (P)	210.5+67.9	70	4
Onapristone (O)	33.4+4.3	60	0
P+O	77+24.9	70	1

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. Progesterone, 10 mg/pellet/21 days; onapristone, 5 mg/pellet/21 days. The treatments with progesterone and onapristone were replaced every three weeks. Tumors were harvested after 8 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections. Lymph nodes near the cell injection sites were processed for histological analysis. Invasion of TKS-7 breast cancer cells to lymph node was clearly seen in four tumor injections in progesterone-treated mice. No lymph node metastasis were observed in either control or onapristone-treated mice; and only one lymph node metastasis was identified in progesterone- and onapristone-treated mice. The lymph node metastasis was defined by the appearance of the small island or foci of breast cancer cells which are bigger and lighter (hematoxylin stain) than the lymphatic cells (see Fig. 1C, p42).

Fig. 4. Effects of progesterone and tamoxifen on TKS-7 tumor growth



Effects of progesterone and tamoxifen on TKS-7 tumor growth

	Tumor size (mm^3 + SD)	Tumor incidence (%)
Control	33.4+16.7	40
Progesterone (P)	134.5+36.3	70
Tamoxifen (T)	83.6+23.8	100
P+T	259+65.9	100

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. Progesterone, 10 mg/pellet/21 days; tamoxifen, 5 mg/pellet/60 days. The treatments with progesterone were replaced every three weeks. Tumors were harvested after 6 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections.

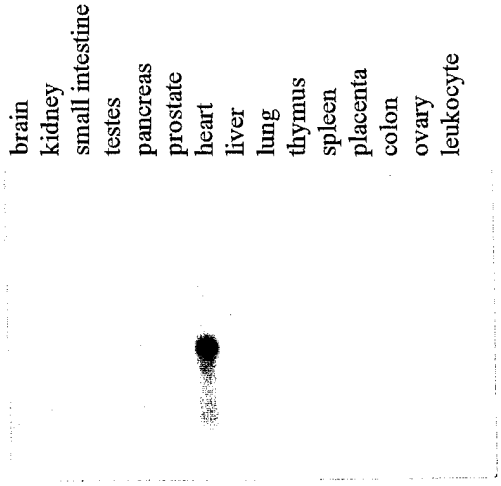
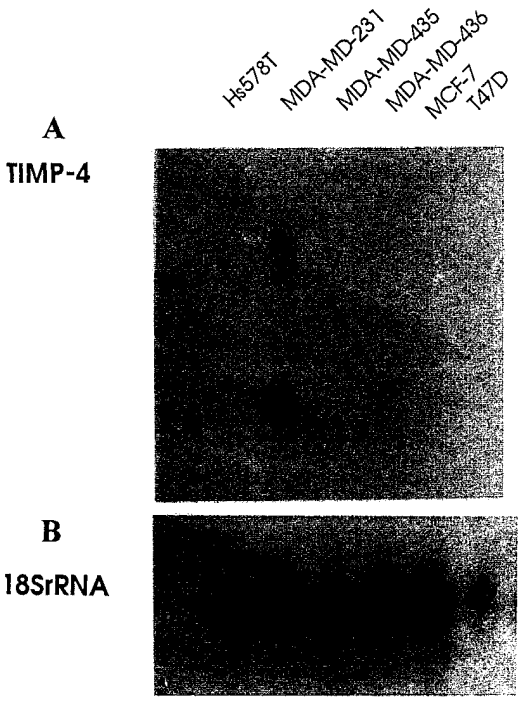


Fig. 6 The expression of TIMP-4 gene in a variety of normal human adult tissues. 20 ug of total RNA from each of the above tissues was analyzed in Northern bolt using a random primer probe. A strong hybridizing band of about 1.4 kilobase was recognized in the lane corresponding to RNA from adult heart. Additional bands corresponding to mRNA species of about 4.2 kb, 3.2 kb and 2.4 kb were also detected in this tissue. Similar bands, although with clear differences in their relative intensity, were also obtained in kidney, placenta, colon and testes. By contrast, none of them was present in other specimens analyzed like liver, brain, lung, thymus and spleen. Several possibilities could contribute to explain the occurrence of multiple TIMP-4 transcripts, including alternative utilization of polyadenylation sites, variable extension of the 5' flanking region or existence of additional genes with a high degree of sequence similarity with TIMP-4. Interestingly, the presence of multiple transcripts in both normal and tumor tissues has been reported for TIMP-2 [75] and TIMP-3 [49].



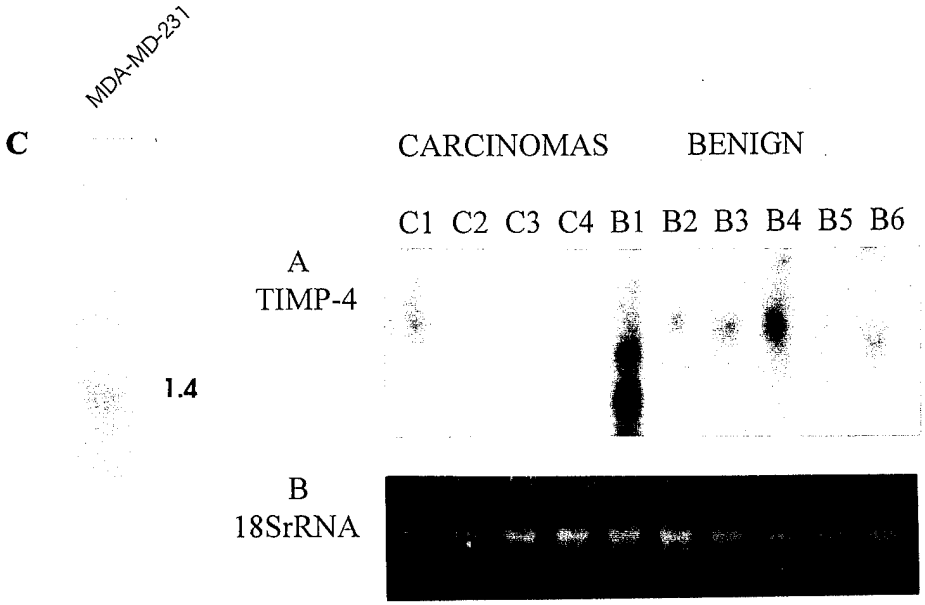


Fig. 8 Northern blot analysis of TIMP-4 expression in breast cancer cells. RNAs from above breast cancer cell lines were isolated and subjected to Northern blot analysis by hybridization with the full-length cDNA (A) or with a 390 bp anti-sense RNA probe corresponding to the 3'-end of the gene (C); the antisense probe is a 390 bp extending from nucleotides 800 to 1190 (the end of the 3'-end of the cDNA) The probe covers 93% of the entire 3' untranslated region, and was generated by PstI cut of the Bluscript vector followed by RNA synthesis with T7 polymerase. The integrity of the RNA was ascertained by direct visualization of the 18 S rRNA in stained gel (B).

Fig. 9 Northern blot analysis of TIMP-4 in human breast tissues. Total RNAs were prepared from six metastatic breast carcinomas (C for carcinomas) and four benign breasts (B for benign breast). RNA samples from B2-B6 were isolated from breast fibroadenomas, and RNA sample of B1 was isolated from breast hyperplasia. Each lane contained 30 ug of total RNA. (A) TIMP-4 RNA hybridized with ^{32}P-labeled full-length TIMP-4 cDNA probe. (B) 18 S rRNA indicating the integrity of the RNA samples and the loading control.

Fig. 7

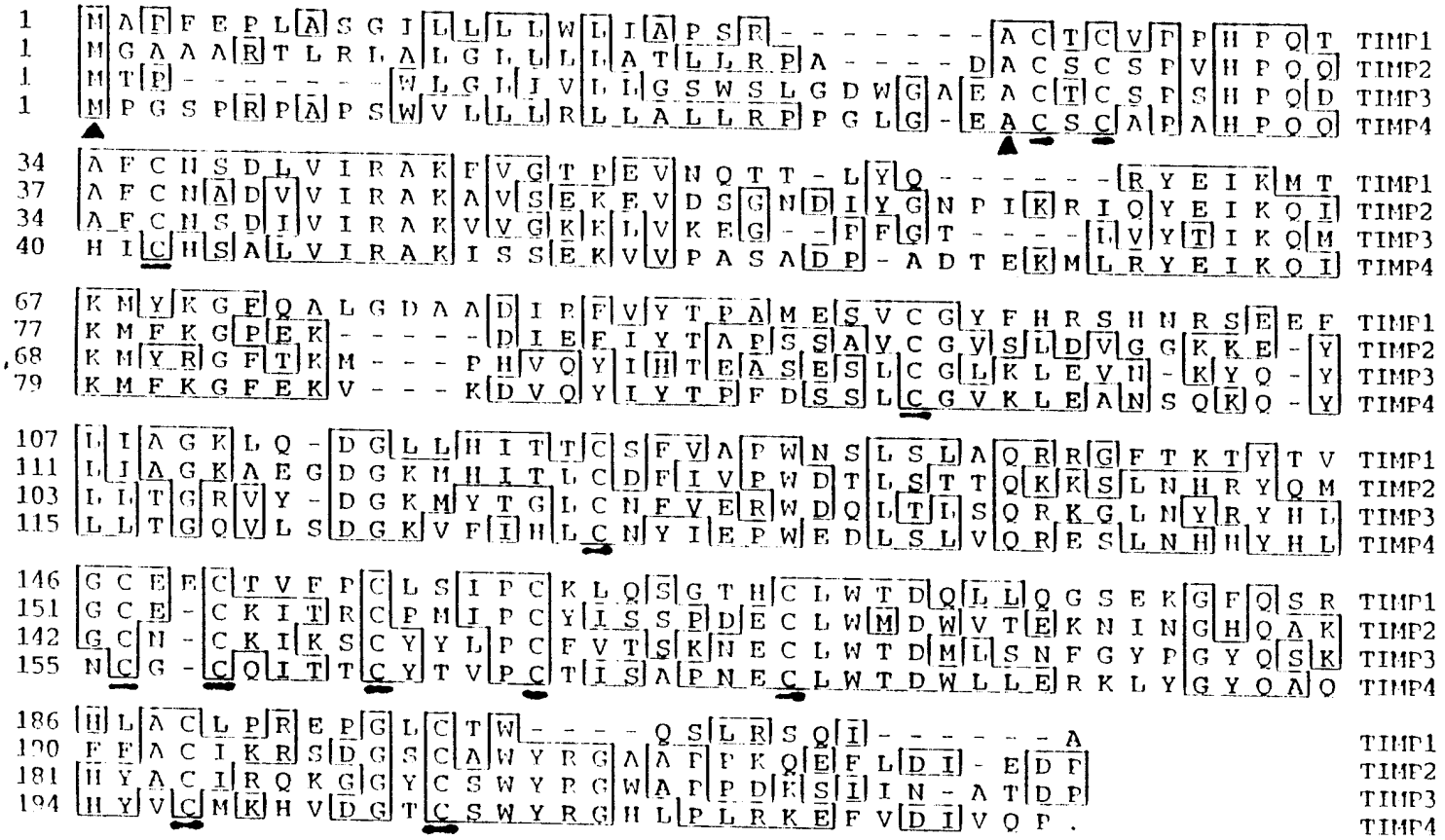


Fig. 7. Comparison of the predicted amino acid sequence of human TIMP-4 with human TIMP-1, TIMP-2, and TIMP-3. The amino acid sequence of TIMPs 1, 2 and 3 were obtained from SwissProt and aligned with the TIMP-4 deduced sequence using the clustal method of the MegAlign Program from the DNASTAR software package. Conserved bases are boxed, the 29 amino acid putative signal sequence is shown between two triangles (▲), and 12 conserved cysteine residues are underlined.

Fig. 10

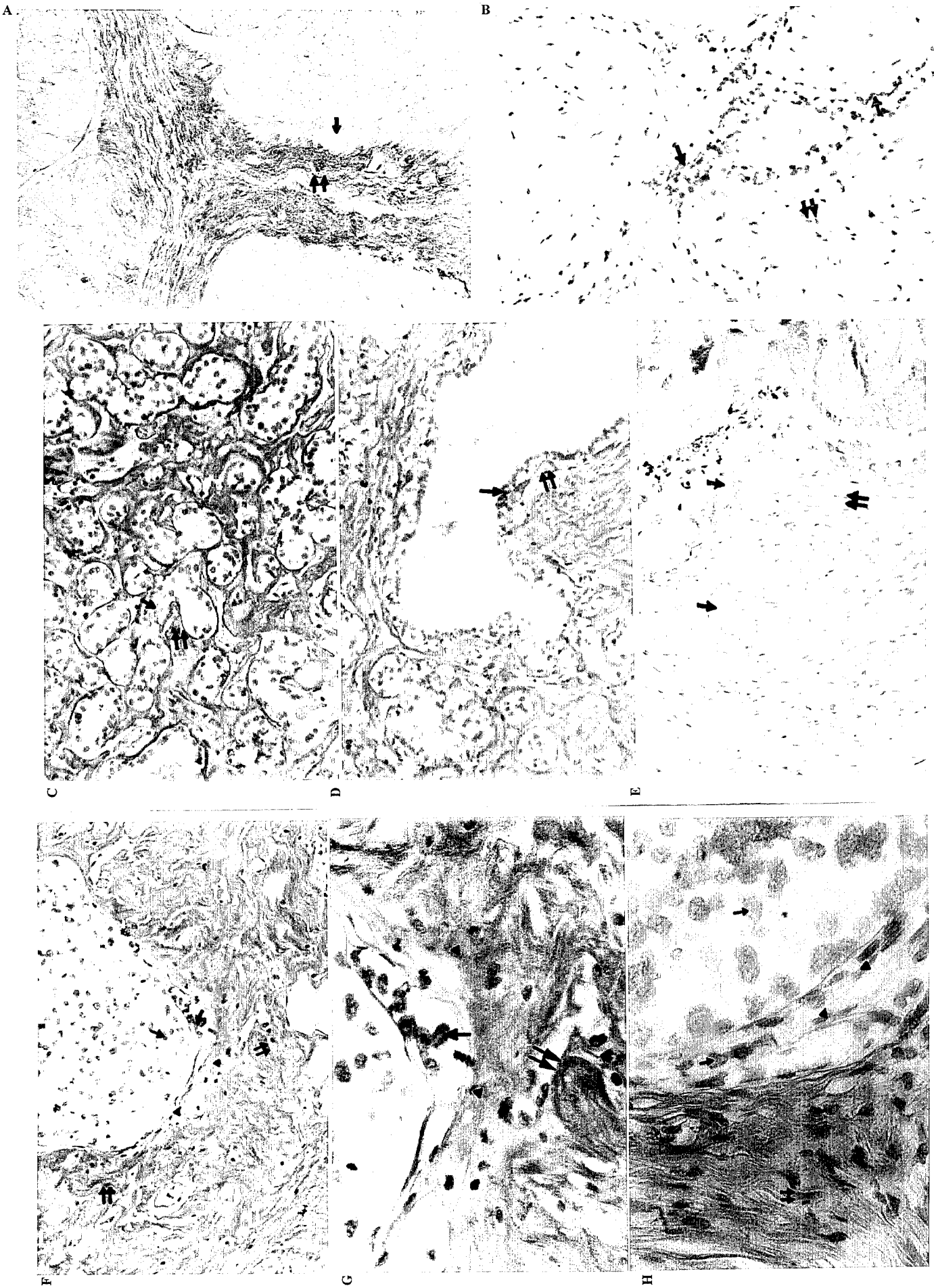
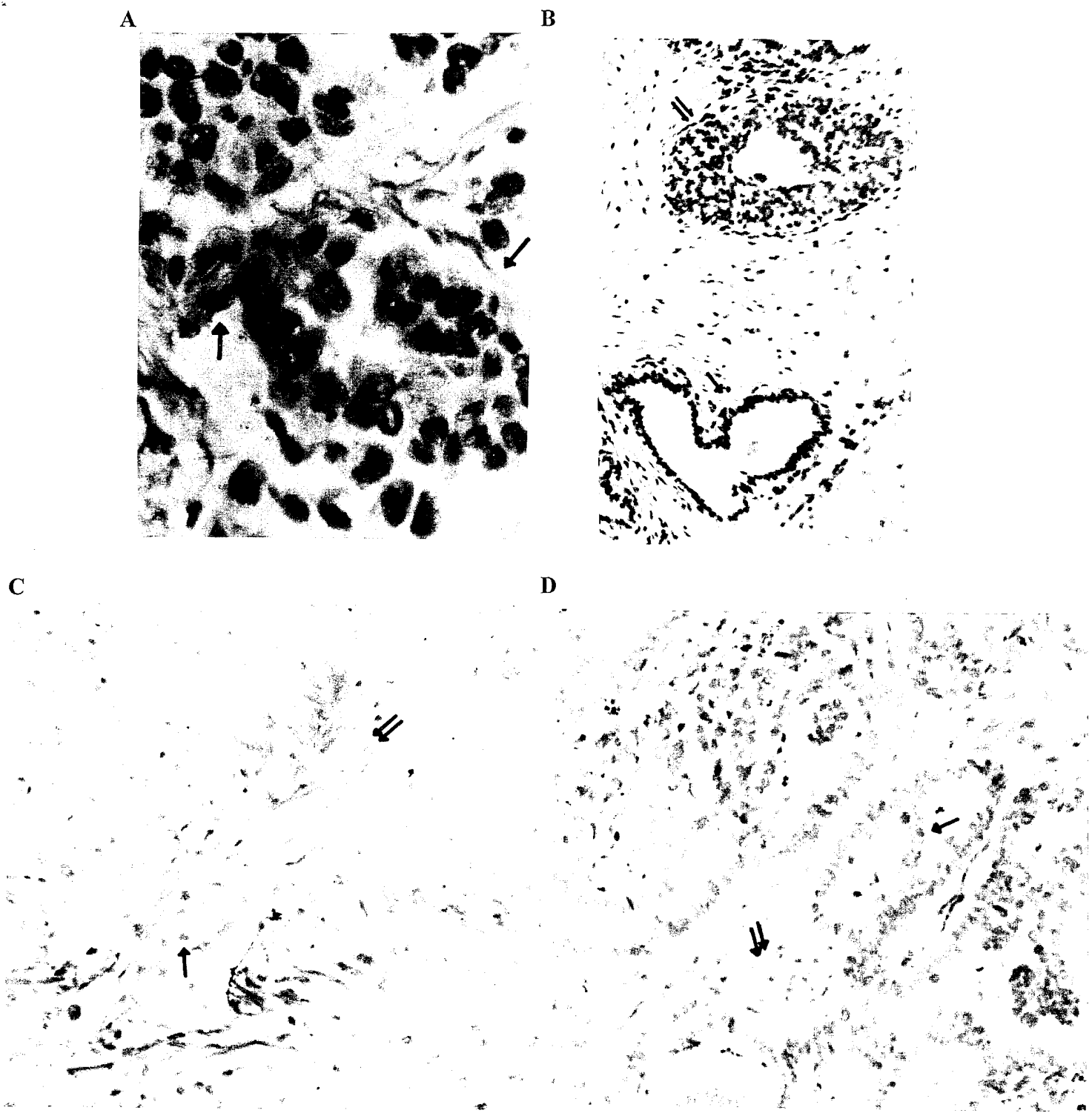


Fig. 11



See next page for figures 10 & 11 legends

Fig. 10. *In situ* hybridization analysis of TIMP-4 (A-H) expression in human breast. Double arrows indicate the stromal cells and single arrow indicate both normal and neoplastic breast epithelial cells. Areas with brown color indicate the TIMP-4 signals. **(A)** Low magnification (100X) view of fibroadenomas showing a strong labeling of stromal cells for TIMP-4 mRNA; breast epithelial cells are negative. **(B)** Low-power view (100X) of fibroadenoma with a sense probe as the negative control; no significant labeling above background was observed under similar conditions using a sense riboprobe. **(C)** Breast hyperplasia shows strong TIMP-4 expression in stromal cells surrounding lobular epithelial cells; **(D)** Positive staining in stromal areas of normal breast duct. **(E)** Low magnification of infiltrating breast carcinoma shows negative staining for both neoplastic breast epithelial cells and surrounding stromal cells; **(F)** *In situ* ductal carcinoma; low magnification view (100X) the strong positive TIMP-4 staining in stromal cells not close to neoplastic cells (double arrows); but stromal cells immediately surrounding the neoplastic cells (▲) were negative or very weakly stained for the TIMP-4 expression. **(G)** High magnification (400X) view of the same *in situ* carcinoma of section F. Please note the negative or very weak TIMP-4 staining in the stromal areas immediately surrounding the neoplastic cells **(▲). (H)** Another *in situ* carcinoma showing a strong TIM-4 staining in the stromal areas not close to the neoplastic cells but a very weak TIMP-4 staining in the stromal ares immediately surrounding the neoplastic cells. Please notice that some neoplastic cells (➞) already invade into the nearby stromal area. All the Sections were counterstained lightly with hematoxylin.

METHODS. In situ hybridization was carried out as described in ref. 76. Deparaffinized and acid-treated sections (5-um thick) were treated with proteinase K, pre-hybridized, and hybridized overnight with digoxigenin labeled anti-sense transcripts from a TIMP-4 cDNA insert. The TIMP-4 antisense probe is a 390 bp, covering 93% of entire 3' untranslated region as described in Fig. 4. Hybridization was followed by RNase treatment and three stringent washings. Sections were incubated with mouse anti-digoxigenin antibodies. The colorimetric detection were performed using a standard indirect immunoreaction with the secondary antibodies conjugated with biotin by DAKO's Universal LSAB Kit according to manufacturer's instructions.

Fig. 11. *In situ* hybridization analysis of TIMP-3 (A,B) and TIMP-2 (C,D) expression in human breast. **(A)** high magnification (400X) of infiltrating breast carcinoma showing a labeling of neoplastic breast epithelial cells (arrow) for TIMP-3 mRNA; **(B)** low magnification view (100x) of negative staining of TIMP-3 mRNA in either normal duct (arrow) or hyperplasia (double arrow); **(C)** positive staining of TIMP-2 in the stroma cells (arrow) surrounding the infiltrating breast carcinoma; (D) a negative or very weak TIMP-2 staining in benign breast hyperplasia. All sections were counterstained lightly with hematoxylin.

METHOD. *In situ* hybridizations were carried out as described in Fig. 5. Sections were incubated with digoxigenin-labeled anti-sense transcripts from a TIMP-3 cDNA insert (A,B) or TIMP-2 anti-sense probe (C,D). The TIMP-3 antisense probe is a 1273 bp, representing entire 3' untranslated region. The probe was generated by PstI cut of TIMP-3 cDNA plasmid and followed by T7 polymerase. The template for TIMP-2 riboprobe was NotI-linearized PBS-TIMP-2, which produced approximately 1.1 kb probe. Sections A and B were incubated with mouse anti-digoxigenin antibodies. The colorimetric detection were performed using a standard indirect immunoreaction with secondary antibodies conjugated with biotin by DAKO's Universal LSAB Kit according to manufacturer's instructions. Section C was subjected to autoradiography using NTB2 emulsion (Kodak) for 18 days followed by developing.